

Exhibit A

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Direct introduction of genes into rats and expression of the genes

(transfection)

NISSIM BENVENISTY AND LEA RESHEF

Department of Developmental Biochemistry, Institute of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

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ABSTRACT A method of introducing actively expressed genes into intact mammals is described. DNA precipitated with calcium phosphate has been injected intraperitoneally into newborn rats. The injected genes have been taken up and expressed by the animal tissues. To examine the generality of the method we have injected newborn rats with the chloramphenicol acetyltransferase prokaryotic gene fused with various viral and cellular gene promoters and the gene for hepatitis B surface antigen, and we observed appearance of chloramphenicol acetyltransferase activity and hepatitis B surface antigen in liver and spleen. In addition, administration of genes coding for hormones (insulin or growth hormone) resulted in their expression.

DNA-mediated gene transfer is an important tool for studying the function of genes and their control. Various techniques involving transfection [either by precipitation of DNA with calcium phosphate (1) or in the presence of DEAE-dextran (2)], transduction [using retroviruses as vehicles (3)], and direct microinjection (4) have been used to introduce genes into cells in culture. Transfection results in the insertion of many copies of the DNA which, while persisting in the cell, are transiently expressed. Alternatively, the foreign DNA may integrate into the cell genome and thus be permanently expressed and inherited by the next generation. Transient and stable expression have been complementary in identifying regulatory elements in the DNA. However, introduction of DNA into terminally differentiated cells has encountered difficulties (5), and it may be incorrect to apply conclusions drawn from studies in cultured cells to the regulation of gene expression in the whole animal. Therefore, methods have been sought to introduce genes into animals *in vivo*.

Several attempts have been made to administer genes into mammals. Microinjecting genes into fertilized oocytes has produced transgenic animals (for review see ref. 6). Alternatively, attempts have been made to introduce genes into mature animals by transplantation of transformed cells (7, 8), by injecting genes entrapped in liposomes (9), by infecting with virus (10), and by injecting DNA directly into specific organs (11). All, except the first method, attempted to introduce foreign DNA into tissues of the recipient animals. Recently, calcium phosphate-precipitated DNA, containing polyomavirus sequences, has been injected directly into the liver and spleen of mice. DNA in intact supercoiled form has been found, depending on its ability to replicate (11). We have examined the potential expression of genes introduced *in vivo* by injecting calcium phosphate-precipitated DNA intraperitoneally.

MATERIALS AND METHODS

Animals. Newborn Sabra rats from the Hebrew University breeding center have been used (except 3-week-old rats in

one experiment as indicated in the text). Streptozotocin-diabetic newborns were produced as described (12).

Preparation of Calcium Phosphate-Precipitated DNA and Its Injection *in Vivo*. Supercoiled plasmid DNA, dissolved in 0.5 ml of 125 mM CaCl₂, was added to 0.5 ml of 2× Hepes-buffered saline (280 mM NaCl/1.5 mM Na₂HPO₄/42 mM Hepes, pH 7.1) with continuous nitrogen bubbling. After 30 min at room temperature the precipitate was collected by 2-min centrifugation at 12,000 × g. The precipitate was gently dispersed in a small volume of saline and injected intraperitoneally.

Assay of Chloramphenicol Acetyltransferase (CAT) Activity. Liver and spleen were homogenized in a buffer containing 0.25 M Tris-HCl, pH 7.5, and CAT activity was determined in the 12,000 × g supernatant (13) using 400 μg of tissue protein.

Genomic DNA Preparation. DNA from rat liver and spleen was extracted from the tissues according to Hewish and Burgoyne (14, 15).

Restriction Enzyme Digestion and Southern and Dot-Blot Hybridization Analysis of the DNA. Southern blot hybridization analysis (16) was performed with GeneScreenPlus membranes. Prehybridization and hybridization were carried out under conditions recommended by the supplier, using appropriate DNA probes ³²P labeled by nick-translation (17).

Blot and Dot-Blot Hybridization Analysis of RNA. Liver total RNA was extracted and centrifuged through a CsCl cushion according to Chirgwin *et al.* (18) to separate the RNA from DNA. The RNA was analyzed by blot and dot-blot hybridization (15, 19).

Recombinant DNA Used for Injections and as Molecular Probes. The recombinant supercoiled DNA pSV1cat (13), pSV2cat (13), pRSVcat (20), and pAd12cat (21) are plasmids harboring the CAT gene fused with the respective viral promoters. pCAP is a recombinant plasmid containing the structural CAT gene fused to a 545-base-pair *Bam*HI-*Bgl*II fragment of the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter (22). pCA9 is a similar plasmid in which the PEPCK promoter has been inserted in the reverse orientation. pLSV-HBsAg is a recombinant plasmid (23) containing a truncated gene for hepatitis B surface antigen (HBsAg) fused to simian virus 40 early promoters. Human growth gene is a 2.6-kilobase (kb) *Eco*RI genomic fragment of the growth hormone gene cloned in pBR325 (24). Mouse preproinsulin gene is a 1.3-kb *Eco*RI genomic fragment cloned in pUC18 (25).

***In Situ* Immunoperoxidase Staining of HBsAg.** Serial thin sections from different regions were prepared from the tissue after Formalin fixation and embedding in paraffin and were stained for HBsAg by the indirect immunoperoxidase technique (26, 27), using a commercial kit from the Behring Institute (Frankfurt). Antigen existence was revealed by the appearance of small brown granules that filled the cytoplasm.

RESULTS

We have taken advantage of the DNA-mediated gene transfer method, used to transfect cultured cells *in vitro* (1), and applied it to *in vivo* experiments in which the DNA, precipitated in calcium phosphate, was injected into newborn rats. To determine the uptake of such precipitated DNA and its distribution among various tissues, radioactively labeled DNA was injected intraperitoneally (Fig. 1). By 40 hr after injection the radioactivity was found predominantly in the intestine and liver. Subsequently, the material was gradually distributed in all tissues, with a relatively higher concentration in the spleen. By 96 hr a considerable fraction of DNA breakdown products (acid-soluble) was found in the bladder.

Distribution of the radioactivity between nuclear and extranuclear compartments was determined in the liver after separating nuclei from tissue homogenates (28). By 40 hr, 60% of the liver radioactivity was confined to the nuclei, out of which more than 80% was precipitable by trichloroacetic acid and ethanol. By 96 hr, 40% of the liver radioactivity was found in the nuclei and 60% of it was precipitable by ethanol. The radioactivity residing in the extranuclear fraction consisted of 85% acid-soluble material. Distribution of the radioactivity in hepatocytes and nonhepatocytes was determined by separating hepatocytes according to Vedel *et al.* (29). By 48 hr after the DNA injection 40% of the radioactivity in the liver resided in hepatocytes. Injection of free DNA (not precipitated with calcium phosphate) resulted in a rapid degradation and elimination so that by 40 hr most of it, consisting of breakdown products, was concentrated in the bladder.

In a second set of experiments, expression of the injected genetic material has been determined. Using the calcium phosphate precipitate method, we injected 1-day-old neonates intraperitoneally with 10 μ g of DNA of specific genes. The copy numbers and expression of these genes, taken up by the liver and spleen, have been determined 48 hr after injection. For this purpose we have used fused recombinant genes containing the structural prokaryotic gene coding for CAT under the control of eukaryotic viral promoters [pSV1cat (13), pSV2cat (13), pRSVcat (20), and pAd12cat (21)] or the promoter of a liver-specific rat gene, coding for PEPCK (22). Genomic DNA, obtained from liver and spleen of animals injected with pSV1cat and pRSVcat, was analyzed by Southern and dot-blot hybridization using a pSV1cat DNA probe. Copy numbers of these sequences were computed from dot-blot hybridization analyses of 1 and 10 μ g of tissue DNA, in reference to dots of known amounts of plasmid (Fig. 2A). Thus, up to 5×10^8 copies of foreign DNA were revealed in 10 μ g of tissue DNA. Ten micrograms of DNA represents 2 million cells, on the basis of 5 pg of DNA per cell (30). It therefore follows that up to 250 copies of foreign DNA per cell have been taken up by the tissues. Southern blot hybridization analysis (16) revealed intact undegraded sequences of the injected DNA in the animal tissues (Fig. 2B).

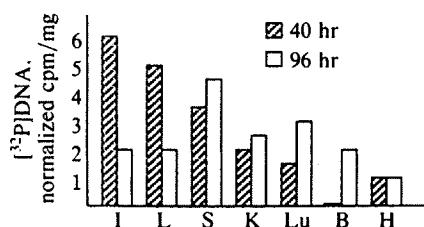


FIG. 1. Distribution of injected radioactive DNA *in vivo*. Radioactivity in each tissue is given relative to its wet weight and normalized to cpm/mg for heart. Tissues: I, intestine (presumably includes residual radioactivity from the injection locus); L, liver; S, spleen; K, kidney; Lu, lung; B, bladder; H, heart.

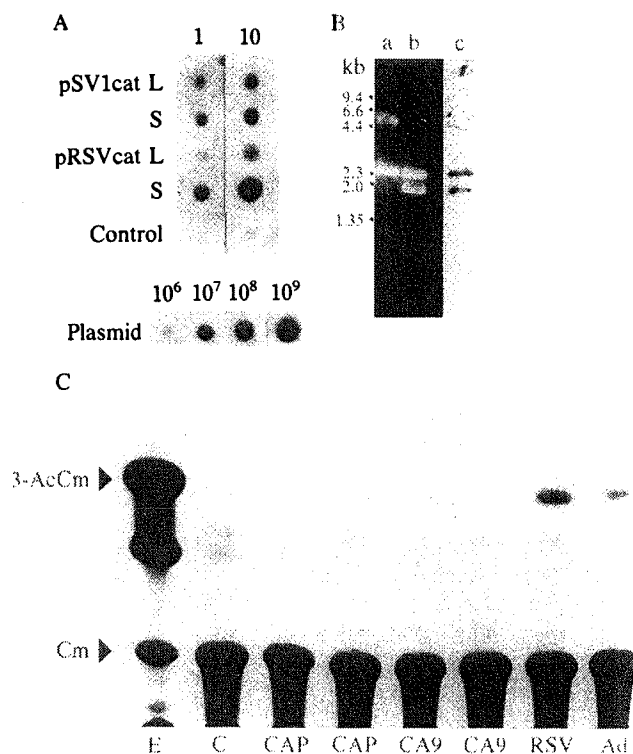


FIG. 2. Copy number and *in vivo* expression of injected genes. (A) Estimation of copy number of the injected DNA. DNA was extracted from newborn rats 48 hr after intraperitoneal injection of the recombinant plasmids pSV1cat or pRSVcat, and 1 or 10 μ g of DNA from liver (L) or spleen (S) was dot blotted and hybridized with pSV1cat [32 P]DNA. Control, liver extract from uninjected animal. Known amounts of pSV1cat DNA, equivalent to 10^6 – 10^9 molecules, were dot blotted (plasmid) as a reference. Autoradiography was for 5 hr. (B) Southern blot hybridization (16) of pRSVcat DNA taken up by the spleen. The ethidium bromide image of plasmid DNA containing pRSVcat before (lane a) and after (lane b) its digestion with *Eco*RI is shown as a reference. Southern blot hybridization of *Eco*RI digest of spleen DNA (10 μ g) from newborn rat injected with this recombinant DNA (lane c) is shown. Autoradiography was overnight. Positions of markers are shown on the left. (C) Activity of rat liver-specific promoter with respect to its orientation: Rat neonates were injected with recombinant CAT genes fused with viral promoters [pRSVcat (RSV) and pAd12cat (Ad)] or with a cellular promoter (for PEPCK) in two orientations [pCAP (CAP) is in the correct orientation and pCA9 (CA9) is in the reverse orientation]. The autoradiogram of the chromatographed assay products is presented. 3-AcCm, 3-acetylchloramphenicol; Cm, unreacted chloramphenicol. Lane E, purified CAT activity; lane C, activity in liver extract from control animal. CAT activities in pmol of 3-acetylchloramphenicol per 100 μ g of liver protein are as follows: C, 0.31; CAP, 0.78 and 0.65; CA9, 0.3 and 0.35; RSV, 1.81; and Ad, 0.95.

CAT activities were detected in the liver and spleen of animals injected with each of the viral promoter-fused CAT genes on top of a background endogenous activity (Table 1). We have noticed that tissue extracts inhibit CAT activity of

Table 1. Detection of CAT activity in liver and spleen

DNA injected	CAT activity*	
	Liver	Spleen
None	0.20	
pSV1cat	0.93	0.70
pSV2cat	0.52	0.48
pRSVcat	1.32	0.66
pAd12cat	2.06	

*pmol of 3-acetyl-chloramphenicol per 100 μ g of protein.

added purified enzyme, suggesting that the CAT activities determined for the injected foreign genes may be minimal values. Nevertheless, the activity of CAT varied apparently in intensity with individual promoters irrespective of the copy number. These findings support the validity of using enzyme activity (CAT) to detect *in vivo* expression of injected genetic material. In addition to viral promoters, we have used a construct of the CAT gene fused with a cellular promoter of the gene coding for PEPCK. CAT activity has been detected in livers of animals injected with this construct (CAP, Fig. 2C). When a construct of the CAT gene fused with this promoter in the opposite direction (CA9) was injected, only the background endogenous CAT activity was detected (Fig. 2C). Thus, expression of foreign genes, introduced by the present method, is dependent on the correct orientation of the promoter.

Variations in the uptake of foreign DNA and its distribution and expression within a tissue were estimated from results of several animals injected with pRSVcat DNA. Uptake and distribution of the DNA in four separate lobes of the liver were determined by Southern blot hybridization (Fig. 3). Two such representative assays demonstrate an even distribution, in all four lobes, in one animal (Fig. 3A) and variation in the distribution of about 3-fold in another animal (Fig. 3B). Similar assays of total liver DNA from an additional three animals (Fig. 3) demonstrated altogether about a 3-fold difference in the amount of DNA taken up by livers of individual animals. CAT activities were likewise determined in four lobes of each liver (mean \pm SD = 1.12 ± 0.06 and 1.04 ± 0.14 pmol of 3-acetylchloramphenicol per 100 μ g of protein in Fig. 3A and B, respectively) and in total liver extracts of four animals (1.32 ± 0.30 pmol/100 μ g of protein).

To directly identify a protein product of the injected gene, we have utilized the cloned gene coding for HBsAg. This is a favorable model because the hepatitis B virus is incapable of infecting this animal species (31), and expression of the gene may be detected *in situ* by immunochemical staining. This assay allows visualization of those cells that are actively engaged in expressing the foreign gene. One-day-old rats were injected with 10 μ g of pLSV-HBsAg DNA (the recombinant plasmid harboring HBsAg gene) in calcium phosphate precipitates. By 48 hr thin sections of liver and spleen of the experimental and control animals were taken for histochemical immunoassay, using specific antibody and indirect immunoperoxidase staining (26, 27). Brown granules indicating existence of HBsAg were visualized in the cytoplasm of different types of liver cells (hepatocytes and nonhepatocytes) of animals injected with the gene. Note the dark granules in the black-and-white photography (Fig. 4). Positively stained cells were likewise observed in the spleen, although they were not as numerous as in the liver (results not shown). However, such expression varied among animals.

Nonspecific control rabbit serum did not reveal any staining. Detailed screening of serial sections from various regions of the liver and spleen of control animals (injected with calcium phosphate alone), did not reveal any positively staining cells (Fig. 4). Thus, the positive cells found in animals injected with the HBsAg gene provided clear evidence for expression of the foreign gene in various cells.

Another approach was to utilize genes coding for hormones and follow their expression. Accordingly, we injected mouse preproinsulin gene and human growth hormone gene into newborn rats. The human growth hormone gene has been tested previously for expression in mouse L cells (1) and in transgenic mice (32, 33). Gene expression at the level of RNA sequences and protein were determined. Three-day-old littermates of normoglycemic and streptozotocin-treated hyperglycemic neonates (12) were injected either with calcium phosphate as control or with the mouse preproinsulin gene in a calcium phosphate precipitate. Expression of the injected gene in the liver was determined by revealing insulin mRNA in this tissue. As shown in Fig. 5A, mouse insulin mRNA, at variable levels, was detectable in all five examined animals. Densitometric scans of the dot-blot hybridization assays enabled estimation of a variation of about 8-fold (SD is \pm 70% of the mean) in the abundance of RNA transcripts among these animals. Although insulin is usually expressed only in the pancreas, when a 1.3-kb genomic DNA fragment encoding mouse preproinsulin was injected, the gene was also expressed in the rat liver. In addition, we have looked for the appearance of insulin itself in the livers of animals injected with the preproinsulin gene. High concentrations of insulin were determined in livers of either normoglycemic or diabetic neonates injected with the gene (265 and 226 microunits per g of liver, respectively, while 56 and 30 microunits per g of liver were determined in animals not injected with the gene). Our results resemble (although on a different time scale) those reported by Nicolau *et al.* (9), who have injected adult rats with rat preproinsulin gene entrapped in liposomes.

We have used our method to administer the human growth hormone gene to 20-day-old rats (10 μ g per animal), and we have detected the respective mRNA in the liver of one of four animals thus injected (Fig. 5B). Blot hybridization analysis revealed a transcript of about 0.9 kb (Fig. 5C), corresponding to the known size of this mRNA (24).

DISCUSSION

Two different approaches have been utilized to introduce foreign genetic material to animals. One involves insertion of the genetic material into fertilized eggs (or embryos) and the other involves insertion into adult organisms. By microinjection of DNA into fertilized eggs (6) or by infection of embryos with recombinant retroviruses (34), successful in-

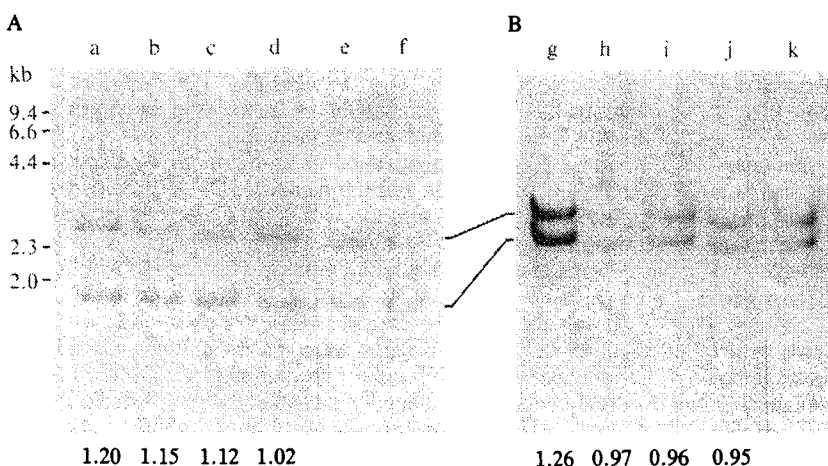


FIG. 3. Distribution of foreign DNA sequences and CAT activities in distinct liver lobes and in several individual animals. DNA and tissue extracts were prepared either from distinct liver lobes or from the whole liver of several individual animals injected with pRSVcat DNA. Southern blot hybridization analysis of genomic DNA (30 μ g) and CAT activities are shown; 0.8% (A) and 1% (B) agarose gels were used for electrophoresis. Lanes a-d and g-j represent assays from distinct liver lobes of two individual animals. Lanes e, f, and k represent assays of DNA from whole livers of three individual animals. Autoradiography was for 5 hr. The numbers underneath the lanes represent CAT activities from each liver lobe.

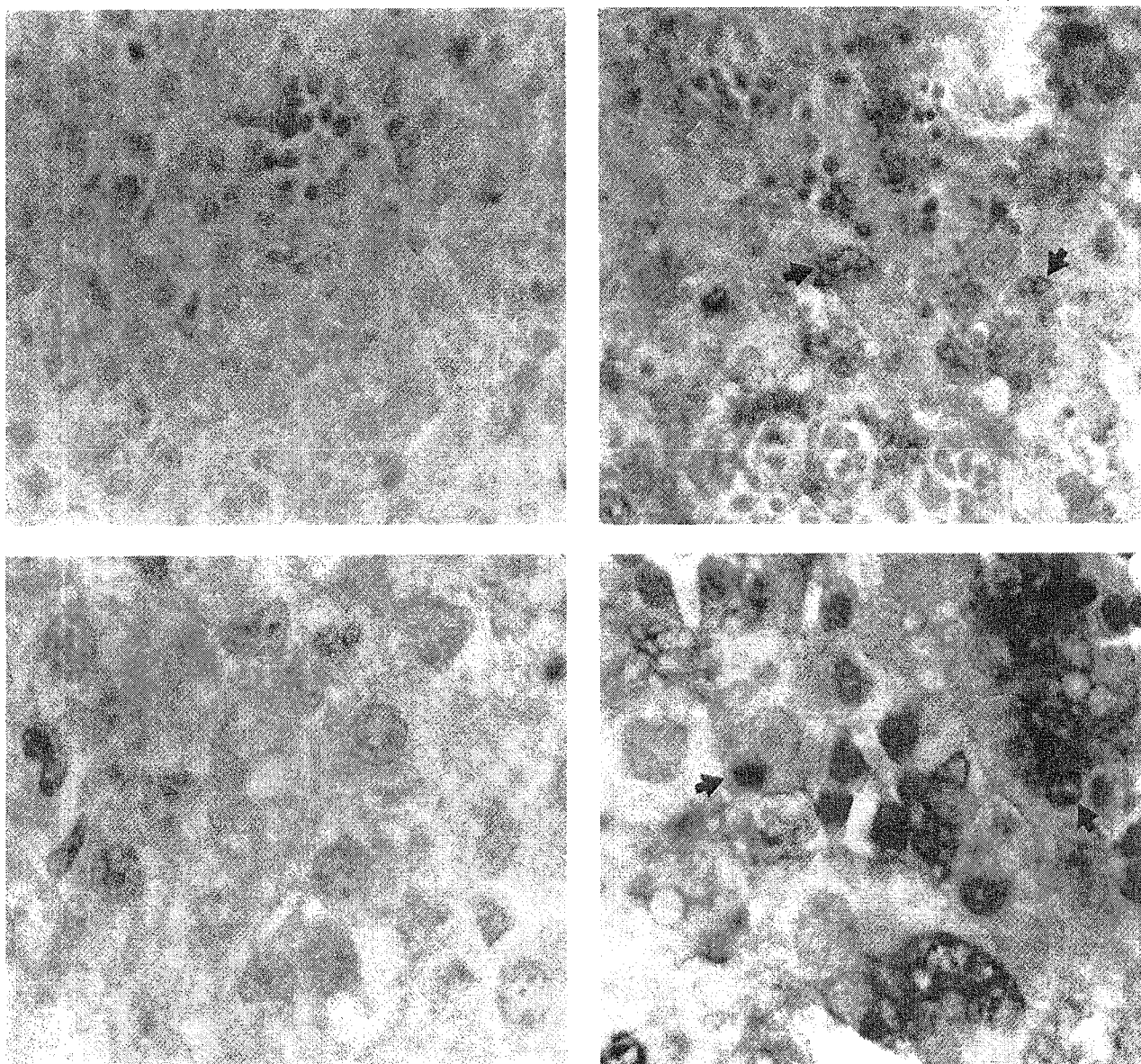


FIG. 4. *In situ* immunoperoxidase staining of HBsAg in sections from newborn liver. Photographs of thin liver sections from control newborn animals injected with calcium phosphate alone (*Left*) and from animals injected with pLSV-HBsAg DNA in calcium phosphate (*Right*). (*Upper*, $\times 930$; *Lower*, $\times 2300$.) Positive immunoperoxidase staining is noted by dark granules in the cytoplasm of cells, as indicated by the arrows.

tegration of the DNA eventually occurs, resulting in the development of transgenic organisms.

Alternatively, insertion of genetic material into adult animals is attractive from the point of view of both basic and applied research. Cells harboring a stably integrated foreign DNA [transfected (7) or infected with retroviruses (8)] have been transplanted into the organism under conditions that select *in vivo* for these cells. Obviously, such a method is limited to actively dividing cells and to tissues capable of such transplantation (for review see ref. 8).

Direct introduction of genetic material (rather than transplantation) seems much less restricted. Along these lines, an attempt has recently been made to infect animals with viruses (10). In this case the herpesvirus served as a vehicle to administer an included bovine growth hormone gene. However, due to tissue tropism, the virus and its inserted gene were confined to T lymphocytes.

Alternatively, taking advantage of liposomes, which are readily absorbed by tissues, a cloned recombinant DNA (preproinsulin gene cloned in a plasmid) entrapped in liposomes has been injected intravenously into rats (9). The DNA was rapidly taken up by the liver and spleen. Yet expression

of the foreign insulin gene, detected by high insulin levels in the liver and spleen, lasted for only 6 hr (9).

Recently, Dubensky *et al.* (11) have succeeded in introducing polyomavirus DNA, in calcium phosphate precipitate, by injecting the precipitate directly into the liver (adult mice) or spleen (newborn mice). They reported a presence of the DNA in the injected organs, which totally depended on its ability to replicate (11). Nevertheless, the fact that such foreign DNA has been preserved intact and has even transiently been replicated was challenging.

These findings encouraged us to examine whether intraperitoneal rather than direct injection of DNA (precipitated in calcium phosphate) into animals would result in its absorption and expression by tissues.

Our results have indeed demonstrated that DNA, injected by the present method, is taken up by tissues, primarily liver and spleen, and is expressed. Direct determination of the abundance of foreign DNA found in the animal tissues gave estimates of up to 250 copies of recombinant DNA per cell. In reality, the DNA may not have been evenly distributed to all cells, as is evident from the immunoperoxidase staining of HBsAg in liver slices *in situ* (Fig. 4). In general, the uptake

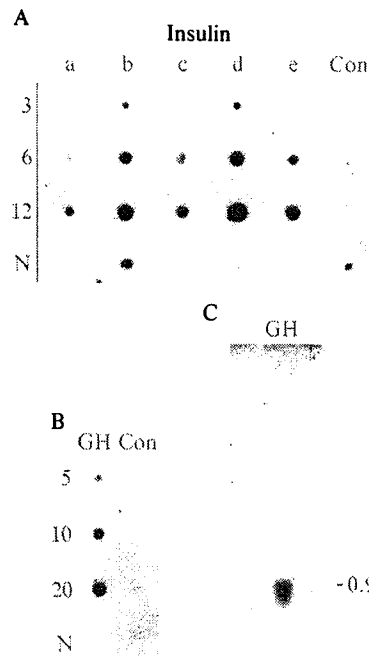


FIG. 5. Expression of injected genes coding for hormones. Dot-blot hybridization analysis of RNA extracted from livers of animals injected with mouse preproinsulin gene (A) or human growth hormone (GH) gene (B). RNA in increasing quantities (3, 6, or 12 μ g from animals a–e in A and 5, 10, or 20 μ g from one animal in B) hybridized with the respective gene probes. Samples labeled N account for traces of DNA that might have contributed to the hybridization. For this, RNA (6 μ g in A and 10 μ g in B) was dissolved in 0.1 M NaOH and boiled for 10 min to destroy the RNA before dot blotting. Control animals (Con) were injected with calcium phosphate alone. Autoradiography was for 12 hr. (C) RNA blot hybridization analysis of human growth hormone transcript: 32 μ g of total RNA from the liver of the positive animal shown in B. One spot, corresponding to about 0.9 kb, was revealed.

of DNA by the liver was not localized, but rather was distributed in all liver lobes (Fig. 3), although the amounts taken up varied by 3-fold among individual animals. By 2 days after injection, the absorbed DNA was found in intact supercoiled form in both liver and spleen. This indicated that it did not integrate in the host genome. Along with the decrease in the peritoneal precipitate, by 4 days after injection, the percent DNA in the material found in the liver also decreased. Thus, the present method enables transient presence of considerable amounts of foreign DNA in the animal tissues. Apart from being intact, such foreign DNA is actively transcribed, as is evident from the insulin and growth hormone transcripts readily detected in the liver. Gene expression is traced to the protein product as shown by the high insulin levels found in the liver, the detectable CAT activity, and the appearance of a new protein, as documented by the presence of HBsAg in tissue slices of the recipient animals.

However, the distribution of the genetic material, introduced by the present method, is predominantly confined to the liver and spleen. Expression of inserted genes is transient and varies considerably among individual animals. This is most clearly demonstrated by the 8-fold difference in the abundance of insulin transcripts among animals and from the fact that growth hormone transcripts were detected in only one out of four animals. In addition, the efficiency of expression of genes introduced by the present method is as

yet unclear. Likewise, it is still unknown whether genes introduced by the present method obey tissue-specific or hormonal control. In addition, it is of interest to examine whether introduction of DNA containing an origin of replication will enable replication and persistence of the foreign DNA in the tissues' nuclei.

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